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Cardiolipin Remodeling in a Chinese Hamster Lung Fibroblast Cell Line Deficient in Oxidative Energy Production

Alison Rusnak,[1] Rick Mangat,[1] Fred Xu,[1] Grant McClarty,[2] and Grant M. Hatch[1]

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The metabolism of cardiolipin was investigated in a Chinese hamster lung fibroblast cell line CCL16-B2 deficient in oxidative energy metabolism and its parental cell line CCL16-B1. Mitochondrial enzyme activities involved in *de novo* cardiolipin biosynthesis were elevated in CCL16-B2 cells compared with CCL16-B1 cells, indicating initially an elevation in cardiolipin biosynthesis. Content of all phospholipids, including cardiolipin and its precursors, and high energy nucleotides were unaltered in CCL16-B2 cells compared to CCL16-B1 cells. When cells were incubated with [1,3-^{3}H]glycerol for up to 4 h radioactivity incorporated into cardiolipin in CCL16-B2 cells did not differ compared with CCL16-B1 cells. In contrast, radioactivity incorporated into phosphatidylglycerol, the immediate precursor of cardiolipin, was elevated over 2-fold in CCL16-B2 cells compared with CCL16-B1 cells. Analysis of the fatty acid molecular species in cardiolipin revealed alterations in the level of unsaturated but not saturated fatty acids in B2 compared with B1 cells. *In vivo* cardiolipin remodeling, that is, the deacylation of cardiolipin to monolysocardiolipin followed by reacylation back to cardiolipin, with [1-^{14}C]palmitate and [1-^{14}C]oleate and *in vitro* mitochondrial phospholipid remodeling with [1-^{14}C]linoleate were altered in CCL16-B2 cells compared to CCL16-B1 cells. Since both the appropriate content and molecular composition of cardiolipin is required for optimum mitochondrial oxidative phosphorylation, we suggest that the difference in CL molecular species composition observed in CCL16-B2 cells, mediated by alterations in *in vivo* cardiolipin remodeling, may be one of the underlying mechanisms for the reduction in oxidative energy production in CCL16-B2 cells.

KEY WORDS: Cardiolipin metabolism; CCL16-B2 cells; mitochondria.

INTRODUCTION

Cardiolipin (CL)[3] is a major mitochondrial membrane phospholipid (Hostetler, 1982). Studies have indicated that CL may be required for the activity of a number of mitochondrial enzymes, several involved in energy metabolism (Kadenbach *et al.*, 1982; Muller *et al.*, 1985; Beleznai and Jancsik, 1989; Beyer and Klingenberg, 1985; Eble *et al.*, 1990; Hoffman *et al.*, 1994; Vik *et al.*, 1981). In mammalian cells, CL is synthesized exclusively in mitochondria via the cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) pathway (Kiyasu *et al.*, 1963; Hostetler *et al.*,

[1] Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3.

[2] Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3.

[3] Abbreviations used: CL, cardiolipin; CDP-DG, cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol; PA, phosphatidic acid; PGP, phosphatidylglycerol phosphate; CMP, cytidine-5'-monophosphate; PG, phosphatidylglycerol; PC, phosphatidylcholine; B1, CCL16-B1; B2, CCL16-B2.

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1971). In this pathway, phosphatidic acid (PA) is converted to CDP-DG by the enzyme PA:cytidine-5′-triphosphate (CTP)cytidylytransferase (Kiyasu *et al.*, 1963). In the isolated rat heart, the conversion of PA to CDP-DG was shown to be the rate-limiting step of CL biosynthesis (Hatch, 1994). The cellular level of CTP was demonstrated to regulate the *in vivo* activity of PA:CTP cytidylyltransferase and hence *de novo* CL biosynthesis (Hatch and McClarty, 1996). In the second step of the pathway, CDP-DG and glycerol-3-phosphate are condensed to form phosphatidylglycerol phosphate (PGP) with the liberation of cytidine-5′-monophosphate (CMP), catalyzed by PGP synthase (Kiyasu *et al.*, 1963). PGP is then rapidly converted to phosphatidylglycerol (PG) by PGP phosphatase. Finally, PG condenses with another molecule of CDP-DG to form CL, with the liberation of CMP, in a reaction catalyzed by CL synthase (Hostetler *et al.*, 1971). Recently, newly synthesized CL was shown to be readily deacylated to monolyso-CL and reacylated back to CL with linoleoyl-Coenzyme A via a monolyso-CL acyl-Coenzyme A acyltransferase in rat liver mitochondrial fractions (Schlame and Rustow, 1990).

Many defects in mitochondrial energy production are associated with alterations in the activity of respiratory chain proteins (for review see Capaldi, 1988). A number of respiration-deficient mutants have been biochemically characterized (for review see Whitfield, 1985). However, limited information is available on the phospholipid metabolism in these mutants. Recently, CL was shown to be essential for oxidative energy metabolism in chinese hamster ovary cells (Ohtsuka *et al.*, 1993). The temperature-sensitive chinese hamster ovary cell mutant PGP-S, with thermolabile PGP synthase, was defective in both PG and CL biosynthesis at the nonpermissive temperature. When mutant cells were placed on a galactose medium, in which 98% of the cellular energy is supplied by oxidative phosphorylation, growth was markedly attenuated compared to wild type cells. This was a result of an impairment in Complex I (rotenone-sensitive NADH-ubiquinone reductase) activity. The mutation in these cells was associated with gross morphological abnormalities. CCL16-B2(B2) is a chinese hamster lung fibroblast cell line deficient in oxidative metabolism and was the first respiration-deficient mammalian cell mutant identified in tissue culture (DeFrancesco *et al.*, 1976; Ditta *et al.*, 1976). These respiration-deficient mutants had morphologically normal mitochondria but exhibited reduced Complex I activity compared to the wild type CCL16-B1 cells (B1). In this study, we examined whether CL metabolism was effected in B2 cells. We show that while the CL content is unaffected, the molecular fatty acid composition and rate of *in vivo* remodeling of CL, that is, the deacylation of CL to monolyso-CL followed by reacylation back to CL, is markedly altered in B2 cells. We further suggest that the alteration in CL remodeling results in an altered CL molecular species composition in B2 cells and that this may be one of the underlying mechanisms responsible for the reduction in oxidative energy production in these cells.

MATERIALS AND METHODS

Materials

[1,3-^{3}H]Glycerol, [5-^{3}H]CTP, [2,8-^{3}H]adenine, [*methyl*-^{3}H]thymidine *sn*-[^{14}C]glycerol-3-phosphate, [1-^{14}C]palmitate, [1-^{14}C]oleate, and [1-^{14}C]linoleate were obtained from Amersham Canada Limited (Oakville, Ontario, Canada). [^{3}H]CDP-DG was synthesized and obtained from Dupont Canada Limited (Missisauga, Ontario, Canada). All lipid standards were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Cell-culture media and reagents were products of Canadian Life Technologies Inc. (GIBCO) (Burlington, Ontario, Canada). Thin-layer chromatographic plates (silica gel G) were from Fisher Scientific (Edmonton, Alberta, Canada). All other biochemicals were of analytical grade and obtained from Sigma Chemical Company.

CCL16-B1 and CCL16-B2 Cells

Cells were obtained from the American Type Culture Collection. They were cultured in Dulbecco's modified eagle medium (high glucose) supplemented with 10% (by volume) fetal bovine serum. Cell cultures were maintained at 37°C saturated with humidified air/5% carbon dioxide. Each dish of cells was subcultured at 1:5 ratio and confluence was usually obtained after 4 days of incubation. All cell incubation procedures were performed at 37°C. For experiments described below each 60-mm dish contained approximately 2×10^6 cells.

Radiolabeling and Harvesting of CCL16-B1 and CCL16-B2 Cells, Extraction and Analysis of Lipids

Cells were incubated for up to 4 h with o.1 μM [1,3-^{3}H]glycerol (10 μCi/dish) or for 4 h with o.1 μM [1-^{14}C]palmitate (0.1 μCi/dish) or 0.1 μM [1-^{14}C]oleate (0.1 μCi/dish). The cells were then washed with 3 ml of ice cold phosphate-buffered saline. Two ml of methanol:water (1:1, by volume) was added to the dish and the cells were removed, using a rubber policeman, into screw cap tubes. The suspension was vortexed twice and a 25-μl aliquot taken for the determination of protein. Two ml of chloroform and 0.5 ml of 0.9% NaCl was added to the suspension which was then vortexed and centrifuged at 1000 × *g* for 10 min. The aqueous phase was removed and the organic phase was washed twice with 2 ml of chloroform:methanol:water (3:48:47, by volume). The aqueous phase was removed and the organic phase dried under nitrogen and resuspended in 100 μl of chloroform:methanol (2:1, by volume). An aliquot of the lipid suspension was placed onto a thin-layer chromatography plate and phospholipids were separated as described (Poorthuis *et al.*, 1976). Lipid spots on the thin-layer plates were visualized by iodine vapor and the silica gel removed and placed into scintillation vials. Five ml of Ecolite$^+$ scintillant was added and the radioactivity determined after a 24 h period. In some experiments the silica gel was removed for the determination of phospholipid phosphorus. In other experiments six dishes of B1 or B2 cells were pooled and CL from these cells isolated by thin-layer chromatography as described above. The silica gel corresponding to these phospholipids was removed and the phospholipid extracted from the gel by washing three times with 3 ml of chloroform:methanol (2:1, by vol). Fatty acid methyl esters of the extracts were prepared and resolved on a Shimadzu GC-14A Gas Chromatograph with an Altech 10% Silar 5CP column.

Enzyme Assays

PA:CTP cytidylyltransferase was assayed by a modification of the method described (Hatch, 1994). A 10% homogenate in 50 mM Tris-maleate, pH 8.5, 0.1 M KCl, 10 mM $MgCl_2$, 0.5% Triton X-100, and 10% glycerol was prepared using tight-fitting Dounce A homogenizer. The homogenate was centrifuged at 1000 × *g* for 2 min (Beckman Model J2-HS Centrifuge with SS-20 rotor) and the resulting supernatant centrifuged at 10,000 × *g* for 15 min. The pellet from this centrifugation was resuspended in homogenizing buffer and designated the mitochondrial fraction. The resulting supernatant was centrifuged at 400,000 × *g* for 15 min (Beckman Model with TLA 100 rotor). The pellet was resuspended in homogenizing buffer and designated the microsomal fraction. To a 16 × 100 mm test-tube (all test tubes were treated with dimethyldichlorosilane, 2% in 1,1,1-trichloroethane) was added in this order, 0.1 mg protein and 0.145 M NaCl to a volume of 60 μl, 10 μl of 0.5 M Tris-maleate, pH 6.5 (Tris buffered with 1.0 M maleic acid solution), 10 μl of 10 mM [5-^{3}H] CTP (specific activity 12,000 dpm/nmole), 10 μl of 0.15 M Triton X-100, and 25 mM PA (prepared by sonicating for 20 min a weighed aliquot of PA in the Triton solution in a silated 16 × 100 mm tube). The reaction was started by the addition of 10 μl of 0.2 M $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30°C for 5 min and terminated by the addition of 0.5 ml of 0.1 M HC1 in methanol. Identification of the [^{3}H]CDP-DG product from the organic fraction was determined as described (Hatch, 1994). To measure conversion of [^{14}C]glycerol-3-phosphate and CDP-DG into lipids, the incubation mixture contained protein (0.1 mg) and 0.145 M NaCl added to a volume of 50 μl, 10 μl of 0.5 M Tris-HCl, pH 7.0, 10 μl of β-mercaptoethanol (prepared fresh), and 10 μl of 5.0 mM [^{14}C-U]glycerol-3-phosphate (specific activity 12,500 dpm/nmole). The reaction was initiated by the addition of 10 μl of 10 mM $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30°C for 20 min and terminated by addition of 0.5 ml of 0.1 M HCl in methanol and subsequent steps performed exactly as described (Hatch, 1994). For assay of CL synthase a 10% homogenate in 10 mM Tris-HCL, pH 7.4, 0.25 M sucrose, and 2 mM EDTA was prepared. The homogenate was centrifuged for 1 min at 1000 × *g* and the resulting supernatant centrifuged at 10,000 × *g* for 15 min. The pellet was resuspended in 1 ml of homogenizing buffer and designated the mitochondrial fraction. CL synthase was assayed in this fraction as described (Hatch, 1994). *In vitro* phospholipid remodeling was measured in mitochondrial fractions prepared from B1 and B2 cells by a modification of the method described (Schlame and Rustow, 1990). The incubation mixture contained 10 μmol Tris-HCl, pH 8.5, 4 μmol β-mercaptoethanol, 90 nmol EDTA, 60 nmol Coenzyme A, 1.2 μmol ATP, 1.4 μmol $MgCl_2$, 2.5 μmol lysophosphatidylcholine, [1-^{14}C]linoleic acid (0.23 μCi/nmole) and 68 μg of protein in a total vol-

ume of 0.3 ml. The mixture was incubated at 37°C for 30 min and terminated by the addition of 3 ml of chloroform:methanol (2:1, by volume). 0.8 ml of KCl was added to initiate phase separation. The organic phase was separated on a thin-layer chromatography plate (Silica gel G) in a solvent system containing chloroform:methanol:water (65:35:5, by volume). The plate was stained with iodine vapor and the spot corresponding to phosphatidylcholine (PC) standard removed and the radioactivity determined as described above.

Other Procedures

Cell viability was assessed using Trypan blue exclusion. Cell protein was determined (Lowry *et al.*, 1951) and phospholipid phosphorus content was measured as described (Rouser *et al.*, 1966). Nucleotides pool sizes were measured as described (Tipples and McClarty, 1993). DNA and RNA synthesis from [2,8-^{3}H]adenine and DNA synthesis from [*methyl*-^{3}H]thymidine were determined as described (Hatch and McClarty, 1996). Student's *t* test was used for the determination of significance. The level of significance was defined as $P<0.05$.

RESULTS

B1 (wild type) and B2 cells were grown in the presence of high-glucose medium. In the presence of high glucose there was no apparent difference in the rate of growth of these cells, in agreement with previous studies (Soderberg *et al.*, 1980; DeFrancesco *et al.*, 1975). In addition, rates of total nucleic acid synthesis from [2,8-^{3}H]adenine and DNA synthesis from [*methyl*-^{3}H]thymidine were determined and were found to be similar between B1 and B2 cells (data not shown).

To investigate if CL metabolism was altered in Chinese hamster lung fibroblast B2 cells, we initially determined the activities of the enzymes of the CDP-DG pathway of CL biosynthesis in B1 and B2 cells. There was no difference in microsomal PA:CTP cytidylyltransferase activity in B2 compared to B1 cells (Table I). Thus, microsomal PA:CTP cytidylyltransferase activity provided a control for a biosynthetic enzyme not affected by the mutation in B2 cells. In contrast, mitochondrial PA:CTP cytidylyltransferase activity was elevated over 2.1-fold in B2 cells compared to wild type cells. In addition, the formation of PG from [^{14}C]glycerol-3-phosphate and CDP-DG (combined PGP synthase and PGP phosphatase activities) was elevated 30% in B2 cells compared with controls. Mitochondrial CL synthase activity in B2 cells was also elevated 2-fold compared with B1 cells. These data initially indicated an elevated production of CL in B2 cells.

Table I. Activity of Enzymes Involved in CL Biosynthesis in CCL16-B1 and CCL16-B2 Cells[a]

	CCL16-B1	CCL16-B2
	pmol/min mg protein[b]	
PA:CTP cytidylyltransferase		
Microsomes	37 ± 2	43 ± 9
Mitochondria	118 ± 16	208 ± 6[c]
PGP synthase and phosphatase	154 ± 24	204 ± 4[c]
CL synthase	3 ± 1	7 ± 2[c]

[a] PA:CTP cytidylyltransferase, CL synthase, and the conversion of [^{14}C]glycerol-3-phosphate and CDP-DG to lipids (PGP synthase and phosphatase activities) were determined as described in Materials and Methods.
[b] Values represent the mean ± standard deviation of at least three separate experiments.
[c] $P < 0.05$.

Cells were then incubated with [1,3-^{3}H]glycerol for up to 4 h and the radioactivity incorporated into PG and CL determined. As seen in Fig. 1A radioactivity incorporated into CL was unaltered in B2 cells compared to B1 cells at all times examined. In contrast, radioactivity incorporated into PG, the immediate precursor of CL, was elevated 2.3-fold by 30 min and 2.6-fold by 120 min of incubation in B2 cells compared to B1 cells, indicating elevated synthesis of PG (Fig. 1B). The *in vitro* increase in mitochondrial PA:CTP cytidylyltransferase activity and formation of PG from [^{14}C]glycerol-3-phosphate and CDP-DG was likely responsible for the increase in radioactivity incorporated into PG in these glycerol cell labeling studies. However, the increase in CL synthase activity in B2 compared with B1 cells indicated that the lowered amount of radioactivity incorporated into CL in the glycerol labeling studies was not due to a decreased conversion of PG to CL.

Since B2 cells have a mutation in oxidative energy metabolism and CL biosynthesis may be regulated by the ATP and CTP level of the cell (Hatch and McClarty, 1996; Cheng and Hatch, 1995), we measured nucleotide pools in B1 and B2 cells. There were no significant changes in ATP, CTP, GTP, or UTP level in B2 com-

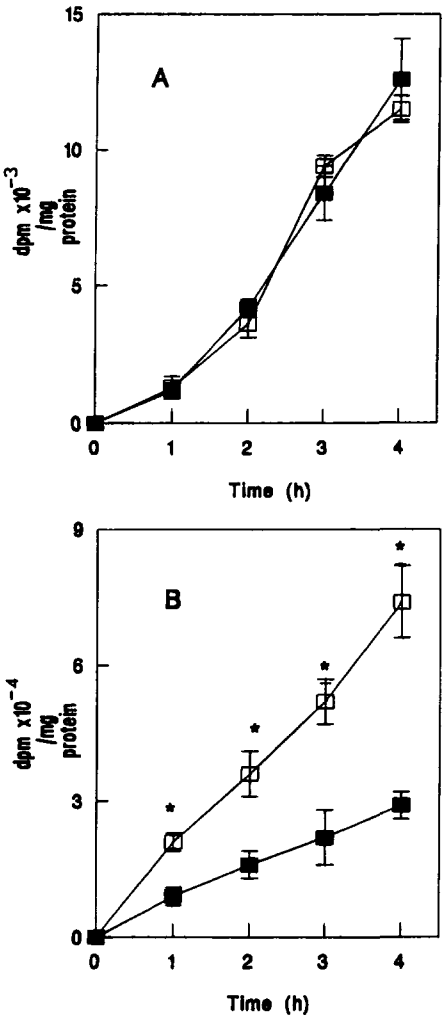

Fig. 1. [1,3-^{3}H]glycerol incorporated into CL and PG in CCL16-B1 and CCL16-B2 cells. CCL16-B1 (solid squares) and CCL16-B2 (open squares) cells were incubated for up to 4 h with [1,3-^{3}H]glycerol and the radioactivity incorporated into CL (A) and PG (B) determined. Values represent the mean ± standard deviation of three separate experiments each of which was performed in duplicate. *$P<0.05$.

pared to B1 cells (Table II). Thus, the lowered [1,3-^{3}H]glycerol incorporated into CL in B2 cells was not due to an alteration in the nucleotide pool size compared to B1 cells. The pool size of CL and phospholipids was examined in B1 and B2 cells and the results are presented in Fig. 2. There were no significant differences in the pool sizes of any major membrane phospholipids, including CL, in B2 compared to B1 cells. The pool size of CDP-DG was 4.1 nmol/mg protein and did not differ between B1 and B2 cells. Thus, the lowered [1,3-^{3}H]glycerol incorporated into

Table II. The Nucleotide Pool Sizes in CCL16-B1 and CCL16-B2 Cells[a]

	CCL16-B1	CCL16-B2
	pmoles/μgDNA[b]	
CTP	145 ± 9	140 ± 14
ATP	794 ± 75	784 ± 97
GTP	211 ± 25	214 ± 26
UTP	238 ± 42	269 ± 22

[a] The nucleotide pool sizes in CCL16-B1 and CCL16-B2 cells were determined as described in Materials and Methods.
[b] Values represent the mean ± standard deviation of at least three separate experiments.

CL in B2 cells was not due to an alteration in the pool size of CL or its precursors.

We then investigated if *in vivo* remodeling of CL was altered in B2 cell. Molecular species analysis of CL in B1 and B2 cells revealed that the principal fatty acid components were palmitic, palmitoleic, stearic, oleic, and linoleic acids (Table III). Interestingly, the

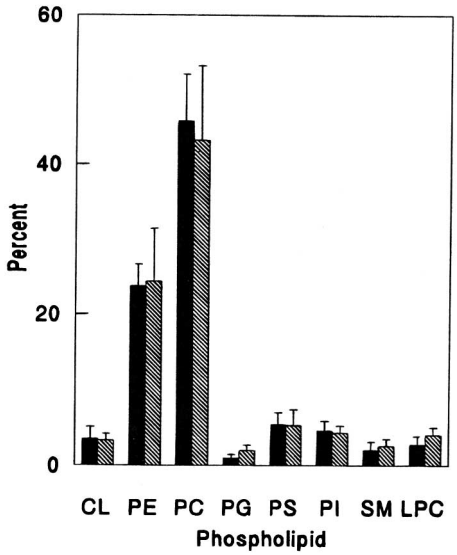

Fig. 2. Phospholipid pool sizes in CCL16-B1 and CCL16-B2 cells. The organic fraction from CCL16-B1 (solid bars) and CCL16-B2 (hatched bars) cells was isolated and phospholipids were separated by two-dimensional thin-layer chromatography and the pool sizes of individual phospholipids determined as described in Material and Methods. CL, cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine; SM, sphingomyelin. Values represent the mean ± standard deviation of three separate experiments each of which was performed in duplicate.

Table III. Fatty Acid Molecular Species of Cardiolipin in CCL16-B1 and CCL16-B2 Cells[a]

Fatty acid	CCL16-B1	CCL16-B2
	Percent in cardolipin[b]	
Palmitic (C16:0)	14.1 ± 2.9	13.4 ± 3.3
Palmitoleic (C16:1)	9.7 ± 1.7	3.1 ± 0.8[c]
Stearic (C18:0)	11.9 ± 1.7	12.5 ± 2.3
Oleic (C18:1)	47.6 ± 3.7	36.8 ± 2.8[c]
Linoleic (C18:2)	13.1 ± 1.9	31.2 ± 2.7[c]
Others	3.6 ± 1.0	3.0 ± 0.9

[a] The organic fractions from CCL16-B1 and CCL16-B2 cells were isolated and CL separated from other phospholipids by two-dimensional thin-layer chromatography. The percent of individual fatty acids in CL was determined as described in Materials and Methods.
[b] Values represent the mean ± standard deviation of three separate experiments.
[c] $P < 0.05$.

Table IV. *In Vivo* Cardiolipin Remodeling in CCL16-B1 and CCL16-B2 Cells[a]

Radioactive fatty acid	Radioactivity incorporated into cardiolipin (dpm/mg protein[b])	
	CCL16-B1	CCL16-B2
[1-^{14}C]Palmitate	88 ± 15	917 ± 98[c]
[1-^{14}C]Oleate	295 ± 15	191 ± 27[c]

[a] CCL16-B1 and CCL16-B2 cells were incubated with [1-^{14}C]palmitate or [1-^{14}C]oleate for 4 h and the radioactivity incorporated into cardiolipin determined as described in Materials and Methods.
[b] Values represent the mean ± standard deviation of three separate experiments.
[c] $P < 0.05$.

percent composition of saturated fatty acids observed in CL (palmitate, stearate) were unaltered between B1 and B2 cells. In contrast, there were marked differences in the percent composition of the unsaturated fatty acids (palmitolate, oleate, and linoleate) in B2 compared with B1 cells. The percent of palmitoleic and oleic acid was significantly reduced 68% and 23%, respectively. The percent of linoleic was significantly elevated 2.4-fold in B2 compared to B1 cells. To determine if these alterations in CL molecular composition were due to alterations in CL remodeling, *in vivo* remodeling of CL in B2 cells was examined. Cells were incubated for 4 h with 0.1 μM [1-^{14}C]palmitate and the radioactivity incorporated into CL determined. [1-^{14}C]Palmitate was chosen since the percent of this saturated fatty acid was similar in B1 and B2 cells (Table III). Thus, a change in radioactive palmitate incorporated into CL would reflect an alteration in CL remodeling. [1-^{14}C]Palmitate incorporated into CL in B2 cells was elevated 10.4-fold compared with B1 cells, indicating a dramatic alteration in the *in vivo* rate of CL remodeling with [1-^{14}C]palmitate (Table IV). Radioactivity incorporated into PG was elevated 6-fold in B2 cells compared with B1 cells (data not shown). We then examined [1-^{14}C]oleate incorporation into CL since the percent of this unsaturated fatty acid was lower in B2 cells compared with B1 cells. [1-^{14}C]Oleate incorporation into CL in B2 cells was reduced 35% compared to B1 cells, indicating a slower rate of remodeling of CL with [1-^{14}C]oleate. Radioactivity incorporated into PG was unaffected in B2 cells compared with B1 cells (data not shown). The results of these studies clearly indicated an alteration in CL remodeling in B2 cells when compared with the wild type B1 cells.

It could be argued that the elevation in [1-^{14}C]palmitate incorporated into CL was simply due to the lack of β-oxidation of fatty acids in B2 cells, resulting in a larger pool of radioactive palmitate available for remodeling of CL. To address this directly, *in vitro* phospholipid remodeling was investigated in isolated mitochondria as described for rat liver mitochondria (Schlame and Rustow, 1990). Mitochondrial fractions isolated from B1 and B2 cells were incubated with [1-^{14}C]linoleic acid and radioactivity incorporated into phospholipids determined. Under these conditions significant radioactivity could not be observed in CL. However, formation of [^{14}C]PC from [1-^{14}C]linoleic acid was 59±22 pmol PC formed/mg protein in B1 cells and increased 2.2-fold ($P<0.05$) to 127±26 pmol PC formed/mg protein in B2 cells. Thus, *in vitro* remodeling of mitochondrial phospholipids was altered in B2 cells compared with wild type B1 cells. Taken together, the above results suggest that one of the mutations in B2 cells resulted in an alteration in CL remodeling.

DISCUSSION

The results or our study clearly demonstrate a dramatic difference in CL metabolism in B2 cells compared to wild type B1 cells. Phospholipids undergo a deacylation–reacylation cycle in order to obtain their appropriate fatty acyl molecular species composition (Lands, 1960). This deacylation–reacylation cycle has been postulated to be responsible for the incorporation

of long-chain polyunsaturated fatty acids into phospholipids (Akesson, 1970). The observed alterations in *in vivo* CL remodeling in B2 cells was likely responsible for the observed modification of the unsaturated fatty acyl molecular species composition in CL compared with the wild type B1 cells. A previous study had indicated that a cycle, comprising CL deacylation and monolyso-CL reacylation by linoleoyl-Coenzyme A, provided a potential mechanism for the remodeling of newly formed CL in rat liver mitochondria (Schlame and Rustow, 1990). These authors demonstrated that when L-palmitoyl-2[^{14}C]linoleoyl-PC was used as acyl donor, liver mitochondria catalyzed the [^{14}C]linoleoyl acylation of monolyso-CL. In agreement with that study we did not observe reproducible incorporation of radioactivity into CL in the presence of [1-^{14}C]linoleic acid alone. However, the observation that [1-^{14}C]linoleic acid incorporation into PC was elevated 2.2-fold in isolated mitochondrial fractions of B2 cells, compared to the wild type B1 cells, supports the notion that mitochondrial phospholipid remodeling was altered in B2 cells.

It was interesting to note that the phospholipid phosphorus pool size of CL and all other phospholipids were unaltered in B2 cells, indicating that the CL content was maintained in spite of an altered remodeling of CL. In B2 cells the increase in enzyme activities involved in CL biosynthesis were likely required to maintain the CL pool size at the level observed in the wild type B1 cells due to the alteration in CL remodeling. One question that should be addressed is why was [1,3-^{3}H]glycerol incorporation into CL in B2 cells unaffected whereas [1,3-^{3}H] glycerol incorporation into PG, the immediate precursor of CL, elevated over twofold? Since the pool size of PG was unaltered in B2 cells the specific radioactivity of PG would be elevated over twofold and an increase in radioactivity incorporated into CL might have been expected. A possible explanation for this discrepancy is that in these cells pre-existing PG may be preferentially utilized for CL biosynthesis.

Many studies have implicated a role for reduced CL levels and an alteration in CL molecular species composition in mitochondrial decay in the aging process (Shigenaga *et al.*, 1994). Rat heart mitochondrial CL and cytochrome *c* oxidase activities were decreased with aging and treatment of these animals with acetyl-L-carnitine restored the normal level of CL in the inner mitochondrial membrane and almost completely restored cytochrome *c* oxidase activity (Paradies *et al.*, 1992, 1994). The mutation in B2 cells appeared to be quite different than that described in Chinese hamster ovary cell PGP-S mutants. The Chinese hamster ovary cell mutant PGP-S exhibited gross morphological abnormalities of mitochondria when CL levels were reduced (Ohtsuka *et al.*, 1993). In contrast, B2 cells appeared to be morphologically normal (DeFrancesco *et al.*, 1975) and CL levels were unaltered (present study). Yet both PGP-S and B2 cell lines had similar deficiencies in Complex I activity (Ohtsuka *et al.*, 1993, DeFrancesco *et al.*, 1975). It is likely that not only the appropriate content of CL but also the appropriate molecular species composition is an important requirement for a functional Complex I (Heron *et al.*, 1977). Moreover, the appropriate content of CL may be required to maintain the structural integrity of mitochondria whereas both the appropriate content and fatty acyl molecular species composition of CL may be required for optimum mitochondrial enzyme activation. In support of the latter hypothesis, the activity of the delipidated rat liver cytochrome *c* oxidase could be reconstituted by the addition of CL (Yamaoka-Koseki *et al.*, 1991). The specific activity of the reconstituted cytochrome *c* oxidase varied markedly and significantly with different molecular species of CL. Indeed, cytochrome *c* oxidase activity was reported to be 199 and 88 μM cytochrome *c* oxidized per min per 10^7 cells in B1 and B2 cells, respectively (Ditta *et al.*, 1976). In a study of patients with Complex I deficiency, muscle cytochrome *c* oxidase activity decreased with age (Scholte *et al.*, 1995). These authors postulated that this was due to peroxidization of the unsaturated fatty acids of CL. In rodents the sensitivity of CL to peroxidation increases with age and this appears to be in part attributable to the replacement of 18:2 with the more readily peroxidizable 22:4 and 22:5 (Langaniere and Yu, 1993). Since CL appears to be essential for oxidative phosphorylation and hence ATP production, it is likely that the differences in CL molecular species composition observed in B2 cells, mediated by an alteration in molecular remodeling, may be one of the underlying mechanisms for the reduction in oxidative energy production in these cells. Thus, CL remodeling enzymes may be linked to the regulation of cellular energy metabolism.

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REFERENCES

Akesson, B. (1970). *Biochim. Biophys. Acta* **218**, 57–70.
Beleznai, Z., and Jancsik, V. (1989). *Biochim. Biophys. Res. Commun.* **159**, 132–139.
Beyer, K., and Klingenberg, M. (1985). *Biochemistry* **24**, 3841–3826.
Capaldi, R. A. (1988). *Trends Biochem. Sci.* **13**, 144–148.
Cheng, P. and Hatch, G. M. (1995). *Lipids* **30**, 513–519.
DeFrancesco, L., Werntz, D., and Scheffler, I. E. (1975). *J. Cell. Physiol.* **85**, 293–306.
DeFrancesco, L., Scheffler, I. E., and Bissell, M. J. (1976). *J. Biol. Chem.* **251**, 4588–4595.
Ditta, G., Soderberg, K., Landy, F., and Scheffler, I. E. (1976). *Somatic Cell Geneti.* **2**, 331–344.
Eble, K. S., Coleman, W. B., Hantgan, R. R. and Cunningham, C. C. (1990). *J. Biol. Chem.* **265**, 19434–19440.
Hatch, G. M. (1994). *Biochem. J.* **297**, 201–208.
Hatch, G. M., and McClarty, G. (1996). *J. Biol. Chem.* **271**, 25810–25816.
Heron, C., Corina, D., and Ragan, C. I. (1977). *FEBS Lett.* **79**, 399–403.
Hoffman, B., Stockl, A., Schlame, M. Beyer, K. and Klingenberg, M. (1994). *J. Biol. Chem.* **269**, 1940–1944.
Hostetler, K. Y. (1982). In *Phospholipids* (Ansell, A. B., and Hawthorne, J. N., eds.), Elsevier, Amsterdam, pp. 215–261.
Hostetler, K. Y., van den Bosch, H., and van Deenen, L. L. M. (1971). *Biochim. Biophys. Acta* **239**, 113–119.
Kadenbach, B., Mende, P., Kolbe, H. V., Stipani, I., and Palmieri, F. (1982). *FEBS Lett.* **139**, 109–112.
Kiyasu, Y., Pieringer, R. A., Paulus, H., and Kennedy, E. P. (1963). *J. Biol. Chem.* **238**, 2293–2298.
Lands, W. E. M. (1960). *J. Biol. Chem.* **253**, 2233–2237.
Laganiere, S., and Yu, B. P. (1993). *Gerontology* **39**, 7–18.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.
Muller, M., Moser, R., Cheneval, D., and Carafoli, E. (1985). *J. Biol. Chem.* **260**, 3839–3843.
Ohtsuka, T., Nishijima, M., Suzuki, K., and Akamatsu, Y. (1993). *J. Biol. Chem.* **268**, 2293–2298.
Paradies, G., Ruggiero, F. M., Petrosillo, G., Gadaleta, M. N., and Quagliariello, E. (1992). *Biochim. Biophys. Acta* **1103**, 324–329.
Paradies, G., Ruggiero, F. M., Petrosillo, G., Gadaleta, M. N., and Quagliariello, E. (1994). *FEBS Lett.* **350**, 213–215.
Poorthuis, B. J., Yazaki, P. J., and Hostetler, K. Y. (1976). *J. Lipid Res.* **17**, 433–437.
Rouser, G., Siakotos, A. N., and Fleischer, S. (1966). *Lipids* **1**, 85–86.
Schlame, M., and Rustow, B. (1990). *Biochem. J.* **272**, 589–595.
Scholte, H. R., Busch, H. F. M., Bakker, H. D., Bogaard, J. M., Luyt-Houwen, I. E. M., and Kuyt, L. P. (1995). *Biochim. Biophys. Acta* **1271**, 75–83.
Schigenaga, M. K., Hagen, T. M., and Adams, B. N. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 10771–10778.
Soderberg, K., Nissinen, E., Bakay, B., and Scheffler, I. E. (1980). *J. Cell. Physiol.* **103**, 169–172.
Tipples, G., and McClarty, G. (1993). *Mol. Microbiol.* **8**, 1105–1114.
Vik, S. B., Georgevich, G., and Capaldi, R. A. (1981). *Proc. Natl. Acad. Sci. USA* **78**, 1456–1460.
Whitfield, C. (1985). In *Molecular Cell Genetics* (Gottesman, M. M., ed.), Wiley-Interscience, New York, pp. 545–588.
Yamaoka-Koseki, S., Urade, R., and Kito, M. (1991). *J. Nutri.* **121**, 956–958.